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The heat responsive wheat *TaRAD23* rescues developmental and thermotolerant defects of the *rad23b* mutant in *Arabidopsis thaliana*

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ABSTRACT

High temperature severely damage the growth and development of crops with climate change. To effectively screen heat responsive proteins in wheat (*Triticum aestivum* L.), the isobaric tandem mass tag (TMT)-labeled quantitative proteomic analysis and quantitative real-time PCR (qRT-PCR) were performed. Here, we found that a wheat RADIATION SENSITIVE 23 protein, TaRAD23, was up-regulated at both protein and RNA levels by exposing to heat stress. Sequence homology analysis indicated that the TaRAD23 is a conserved protein, which is closely related to the *Arabidopsis thaliana* proteins AtRAD23B and AtRAD23A. Genetic knockout of *AtRAD23B,* but not *AtRAD23A,* shows multiple developmental defects, as well as sensitivity to heat stress. Meanwhile, we observed that constitutive overexpression of *TaRAD23* in *rad23b* fully rescued developmental and thermotolerant defects of the mutant. Furthermore, qRT-PCR analysis of heat responsive genes in *rad23b* and its complementary lines suggested that suppression of the heat shock transcription factor *AtHSFA2* and heat responsive genes (*HSP70*, *HSP90*, *HSP17.6* and *HSA32*) may be the cause of the weaker thermotolerance in *rad23b*. Taken together, the data suggest that the heat responsive TaRAD23 is a functionally highly conserved protein that plays an important role in development, as well as the regulation in heat stress response network.

1. Introduction

One of the challenges for global agriculture is the impact of heat stress (HS) on crop production under the increasing global warming, which has the potential to destabilize food security [1]. Predictions of increases in average temperature of 2–5 °C by the end of the century, combined with more frequent heat waves (IPCC 2012), will have severe effects on crop production [2]. Particularly, wheat is one of the important food crops that are easily affected by high temperature. The wheat growing areas in most parts of the world often experience temperatures above 30 °C during anthesis and filling stage, accompanied by dry hot air, which shortens the grain filling period and not only seriously affects wheat processing quality, but also decreases wheat yield by 3.5%– 7.1%. In areas or years with severe HS, production has been reported to reach only 10%–20% of normal values, or to fail completely [3,4].

The heat tolerance of crop is a quantitative character controlled by multiple genes [5,6]. However, due to the lack of an intuitive, stable and reliable evaluation index, the collection and screening of heat tolerance crop varieties is inefficient, making it difficult to breed new thermotolerance crop varieties by conventional methods. It is therefore critical to understand the molecular mechanisms of the plant response to HS (HSR), which is conductive to generate new varieties with improved thermotolerance by genetic engineering. The activation of the HSR is important for plant survival under high temperatures [2,7]. Although HSR is highly conserved among different prokaryotes and eukaryotes organisms, is more complex and has unique features in plants [7]. The heat stress transcriptional factors (HSFs) are the most crucial regulators of the HSR, and they can regulate the transcription of numerous genes participated in signal transduction and metabolic

Abbreviations: HRPs, heat responsive proteins; HS, heat stress; HSF, heat shock transcription factor; HSR, heat stress response; NER, nucleotide excision repair; RAD23, radiation sensitive 23; HSP, heat shock protein; sHSP, small heat shock protein; Ub, ubiquitin; UBA, ubiquitin-associated; UBL, ubiquitin-like; UPS, the ubiquitin/26S proteasome system; WT, wild type

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pathways. Thus, the activity, or inactivity, of these transcription factors directly affects the thermotolerance of plants [7,8]. The activity and composition of the HSF network are regulated by factor-specific and coordinated reactions at multiple levels of gene expression during HSR attenuation. The function and stability of HSF protein involving the chaperone/co-chaperone network is controlled in the same way [2,9,10]. The heat shock binding protein (HSBP), which is a small protein that interferes with HSF oligomerization, is known to regulate some HSFs [11]. The down-regulation of HSF transcript levels is influenced by the alternative splicing (AS) and nonsense-mediated mRNA degradation (NMD) [12–14]. HSF network activity can also be regulated by post-translational regulation, such as post-transcriptional modification (PTM) and protein-protein interactions (PPIs), as well as by HSF abundance, as a consequence of chaperone-mediated ubiquitination and degradation via the ubiquitin (Ub)/26S proteasome system (UPS) [10,15–18].

The UPS is a major cellular system for protein degradation in plants and animals, which controls the level of numerous cell regulators, including transcription factors, by post-translational degradation [19]. The target proteins are first polyubiquitinated by the E1, E2 and E3 enzymes, and then recognized by a set of Ub binding proteins, thus providing strong signals that can be recognized by the 26S proteasome [20,21]. When the ATP-dependent protease complex recognizes appropriately ubiquitylated substrate, the polyubiquitin chains are released from the substrate and disassembled into ubiquitin monomers for reuse, then the unfolded protein is cleaved into small peptides by proteolytic activities in the lumen of the protease complex [22]. The UPS controls numerous processes of a plant's life cycle by way of the tightly controlled degradation of key regulatory proteins, including cell cycle regulation, embryogenesis, self-incompatibility, apoptosis, senescence, photomorphogenesis, intracellular signaling, transcriptional regulation, DNA repair, protein quality control under abiotic and biotic stresses[20,21,23,24].

Ub binding proteins play a vital role in UPS, acting as a bridge between the different parts of the pathway. Several Ub binding proteins are key subunits of the regulatory particle (RP) sub-complex of the 26S proteasome, including RPN1, RPN10 (also named S5a), as well as RPN13 [25–27]. Furthermore, some Ub binding proteins can stabilize and sequester ubiquitylated proteins to prevent proteasomal degradation of interacting proteins and, in other cases, assist transfer them to the 26S proteasome for degradation [24]. These proteins mainly include RADIATION SENSITIVE 23 (RAD23) [28], Dominant Suppressor of KAR1 (DSK2) [29,30], Neuronal-Precursor-Cell-Expressed Developmentally Downregulated Protein 8 (NEDD8) [31], and DNA Damage-Inducible 1 (DDI1) [32]. Amino acid sequence analysis shows that they all have a UBL domain that is homologous to Ub at their N-terminal region, and one or more Ub-associated (UBA) domains in their central and C-terminal regions, which directly bind to the proteasome and Ub conjugates, respectively [22]. These proteins are predicted to mediate the connection of ubiquitylated proteins or UPS substrates with the proteasome through their UBA domain(s) and UBL domain respectively.

Principal shuttles of Ub conjugates are the radiation sensitivity 23 (RAD23) proteins, which act as a bridge between ubiquitylated proteins and the proteasome [19,33]. RAD23 was first identified as a DNA repair factor that participated in the process of nucleotide excision repair (NER) in yeast (*Saccharomyces cerevisiae*) [34]. In this process RAD23 interacts with the DNA damage recognition factor, RAD4, to form a complex, the nucleotide excision repair factor 2 (NEF2), which is responsible for the recognition of photo-lesions. Consequently, mutations in RAD23 family members will result in a hypersensitivity to UV light in yeast [28], severe developmental defects and male sterility in mice [35], and the Xeroderma pigmentosum (one of the light-sensitive skin disease) in humans [36]. Subsequent studies demonstrated that the ubiquitin-like (UBL) domain of yeast RAD23 binds with high affinity to the second Ub-interacting motif (UIM) of the S5a subunit (or RPN10) of the proteasome to felicitate the release of substrates to the 26S proteasome (Hiyama et al.; Mueller and Feigon), and the ubiquitin-associated (UBA)-2 domain of yeast RAD23 is an evolutionarily conserved stabilization signal that protects RAD23 to interact with the 26S proteasome without degradation. Disruption of the UBA2 domain leads to increased sensitivity to UV light in *rad23*Δ yeast mutant, and severe growth defects in *rad23*Δ*/rpn10*Δ double mutant [37]. It has also been demonstrated that Rpn1 binds to RAD23 specifically though the interaction between its leucine-rich-repeat-like (LRR-like) domain and the UBL domain of RAD23 [38]. RAD23, as a ubiquitin receptor, is an accessory component of the 19S proteasome, and has a range of functions outside of NER, for example in reducing manganese toxicity [39], controlling cellular dNTPs production [40], protecting against neurodegeneration [41] and regulating cell death [42]. Furthermore, it has been shown to be involved in protein shuttling to the proteasome during developmental processes, and in response to stress, as well as being connected to the endoplasmic reticulum associated degradation (ERAD) sub-pathway within the UPS [33,43–45].

RAD23 s play multifunctional roles in almost aspects of biological processes, especially in substrate recognition by the UPS, but little is known about plant RAD23 proteins. *Os*RAD23 was the first RAD23 homolog identified in plants by yeast two-hybrid assay [46]. Sturm and Lienhard cloned two carrot (*Daucus carota*) RAD23 homologues and demonstrated that both of the isoforms could complement the UV-sensitive phenotype of *rad23*Δ yeast mutant, indicating that plant RAD23s also involve in the process of DNA damage repair, and possibly through a similar mechanism [47]. Farmer et al. found there are four orthologs of yeast RAD23 in *A. thaliana* and demonstrated that they are functional redundant proteins and only AtRAD23B play a fundamental role in plant growth and development, presumably by delivering ubiquitylated substrates to the RPN10 subunit of the 26S proteasome [48]. More recently, a novel effector protein (SAP54) was found and demonstrated to mediate degradation of the MADS-domain transcription factor (MTF) by interacting with RAD23 during host coercion [49].

However, little is known on the physiological and regulatory functions of RAD23 in normal growth, development, and responses to biotic and abiotic stress. In this study, we found the expression level of a TaRAD23 was increased in filling wheat grains by heat stress. Then we cloned the gene and investigated its function though over-expression it in the *A. thaliana rad23b* mutant. Besides, we firstly found that *rad23b* was sensitive to heat, and heterologous expression of TaRAD23 in *rad23b* not only rescued its developmental defects but also increased its survival rate under heat stress. Further qRT-PCR data indicate that TaRAD23 may be involved in response to heat stress by regulating the expression of *AtHSFA2* and its targets genes. Thus, *TaRAD23* represents a candidate for improving heat tolerance of wheat through genetic engineering, as well as for developing molecular markers to assist breeding selection of heat-tolerant wheat genotypes.

2. Materials and methods

2.1. Plant materials and growth conditions

The CDS sequence of *TaRAD23* was isolated from wheat variety "Chinese Spring". The *A. thaliana* ecotype Columbia-0 (*Col-0*) was used as the wild type (WT). The T-DNA insertion mutant seeds of *rad23b-1* (SALK_103707), *rad23b-2* (SALK_075940) and *rad23a* (SALK_066603) were ordered from NASC (http://nasc.life.nott.ac.uk/) and their genotypes were identified by PCR and RT-PCR (Supplementary Table S1). All the mutants were derived from the *Col-0* ecotype. The growth condition of wheat plants and *A. thaliana* plants was the same as in our previously work [50,51].

2.2. HS treatments and thermotolerance assays

The HS treatments for wheat plants were the same as in our previously work [51]. Three independently biological samples were used

for isobaric tandem mass tag (TMT)-labeled quantitative proteomic analysis. For qRT-PCR analysis of filling wheat grain *TaRAD23* expression under HS, the same conditions were used except for a different day-night temperature: at 15 days after anthesis, the plants were subjected to day/night temperatures of 38/34 °C and a 14/10 h day/night cycle and their filling grains were harvested at 0 h, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h respectively. All the collected materials were stored at −80 °C.

The HS treatments for *A. thaliana* seedlings were as following: 10 days after germination, seedlings were incubated at 38 °C for 90 min in a water bath, then transferred to normal conditions (22 °C, 16/8 h light/dark cycle) for 2 days. After recovery, seedlings were incubated at 45 °C for 2 h, then transferred to normal conditions for another 5 days, photographed and the ratio of green seedlings was calculated as (the number of green seedlings/total number of seedlings) * 100%. All data were quantitatively analyzed using Microsoft Excel software (Microsoft Corporation, www.microsoft.com). The experiments were repeated at least five times. For hypocotyl elongation assay, surface-sterilized seeds were planted in a line on 1/2 MS plates, wrapped in aluminum foil to avoid light and kept at 4 °C for 3 days, then transferred into a growth chamber for vertical culture for 2.5 days in the dark at 22 °C. Later, the plates were incubated at 38 °C for 90 min in a water bath, and followed by 2 h at 22 °C and 45 °C for 90 min, then transferred to growth chamber in the dark at 22 °C. After 3 days, images were photographed, the length of hypocotyls were measured by ImageJ software. The experiment was repeated at least three times and at least three replicates per experiment. For lateral roots assay, surface-sterilized seeds were planted in a line on 1/2 MS plates and kept at 4 °C for 3 days, then transferred into a growth chamber for vertical culture for 5 days to normal conditions (22 °C, 16/8 h light/dark cycle). Later, the plates were incubated at 38 °C for 90 min in a water bath, and followed by 2 h at 22 °C and 45 °C for 30 min, then transferred to normal conditions (22 °C, 16/8 h light/dark cycle). After 5 days, images were photographed, the length of roots were measured by ImageJ software, and the number of lateral roots were counted with the aid of a magnifier. The experiment was repeated at least three times and at least three replicates per experiment.

2.3. Isobaric TMT-labelled quantitative proteomic analysis

Protein extraction, trypsin digestion, LC–MS/MS were described in previous study [51], except that in present study, the tryptic peptides were labelled by a 6-plex TMT kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's protocol. Another difference from previous study was that in Mascot search, ta_IWGSC_MIPSv2.2_HighConf_PROTEIN_2014Jul18.fa (293,053 sequences, downloaded from ftp://ftpmips.helmholtz-muenchen.de/plants) was chose as the database in present work. Accompanied with the labelling kit, Carbamidomethyl (C), TMT6plex (N-term), TMT6plex (K) were set for fixed modification, and Oxidation (M) for variable modification. Only proteins with at least two unique peptides were further quantified, quantitative proteins with a fold change > 1.2 and *t*-test *p* value < 0.01 were assigned as HRPs. The MS data had been deposited to the ProteomeXchange Consortium via the PRIDE [52] partner repository with the dataset identifier PXD006324 and Doi:10.6019/PXD006324 (the reviewers could log into the account with username: reviewer51346@ebi.ac.uk and password: VKicOzZP).

2.4. Vector construction and transformation

TaRAD23 cDNA was amplified by PCR and the primers are listed in Supplementary Table S1. The amplification was made using the PrimeSTAR HS DNA Polymerase (Takara, Dalian, China) and cloned into the pDONR™/Zeo entry vector using BP recombination clonase (Invitrogen, Carlsbad, CA, USA). After the sequence was confirmed, the *TaRAD23* cDNA was transferred to the pBIB-BASTA-35S-GWR destination vector using the LR recombination clonase (Invitrogen, Carlsbad, CA, USA). *E. coli* strain DH5-α was used in all experiments for sub-cloning. The *Agrobacterium tumefaciens* strain GV3101 was used for plant transformation via the floral dip method [53]. Then homozygous lines were selected from the primary transformants. Two independent and complementary homozygous 35S-*TaRAD23/rad23b* lines were selected for further experiments.

2.5. RT-PCR and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted by RNAiso Plus (Takara, Dalian, China) and treated with RNase-free DNase I (Takara, Dalian, China). 1.0 μg RNA was used for first-strand cDNA synthesis by the PrimeScript RT reagent kit (Takara, Dalian, China). qRT-PCR was performed in 96-well blocks with the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) by using the SYBR Premix Ex Taq II kit (Takara, Dalian, China). Actin mRNA was used as an internal control and the comparative threshold cycle (Ct) method was used to determine relative transcript levels for each reaction. Each experiment had three biological replicates and three technical replicates. All primers used for RT-PCR and qRT-PCR are listed in Supplementary Table S1.

2.6. Phylogenetic analysis

The TaRAD23 sequence was used as a query to search the UniProt database (http://www.uniprot.org/). A selection of the identified RAD23A/B/C/D from prokaryotes and eukaryotes was aligned using ClustalX 2.0 software [54]. The neighbor-joining method (1000 bootstrap replicates) was used to generate phylogenetic trees, and MEGA 5.0 software [55] was used for drawing the phylogenetic trees.

2.7. Accession numbers

RAD23A_HUMAN, P54725; RAD23B_HUMAN, P54727; RAD23A_BOVIN, A3KMV2; RAD23B_BOVIN, Q29RK4; RAD23A_MOUSE, P54726; RAD23B_MOUSE, P54728; RAD23A_RAT, A0A0G2K7M2; RAD23B_RAT, Q4KMA2; RAD23_CALJA, F7ISF6; RAD23_DROME, Q9V3W9; RAD23A_ARATH, Q84L32; RAD23B_ARATH, Q84L33; RAD23C_ARATH, Q84L31; RAD23D_ARATH, Q84L30; RAD23_MAIZE, B6T711; RAD23_DICDI, Q54LV1; RAD23_SOLLC, Q9STA6; RAD23_YEAST, P32628; RAD23_NANGC, K8Z3D9; TaACTIN, Q6I6J8; AtTUBULIN, AT1G20010; AtACTIN, AT3G18780; AtHSFA2, AT2G26150; AtHSFA3, AT5G03720; AtHSFB1, AT4G36990; AtHSP90.1, AT5G52640; AtHSP70, AT3G12580; AtHSP17.6, AT5G12020; AtHSP18.2, AT5G59720; AtHSA32, AT4 G21320; AtROF1, AT3G25230.

3. Results

3.1. TaRAD23 is up-regulated by heat stress

To identify important proteins involved in heat stress tolerance in wheat, we screened for heat responsive proteins (HRPs) by exposing the plants to high temperature at grain filling stage and harvested the filling grains for TMT-labeled quantitative proteomic analysis. Among the HRPs, TaRAD23 was identified as 1.6-fold up-regulated in the filling grains by heat stress (Fig. 1A). To complement the relative protein expression of TaRAD23 from the proteomics, qRT-PCR of *TaRAD23* were performed using RNA samples derived from filling wheat grains exposed to heat shock at 38 °C/34 °C for 0 to 48 h. The expression of *TaRAD23* was mainly up-regulated by 3 h of heat shock, declining after that, while still being up-regulated in comparison with the control (0 h) until the 48 h time point (Fig. 1B). Taken together, TaRAD23 was up-regulated by heat stress both at the RNA and protein level, suggesting its important function in heat stress response.

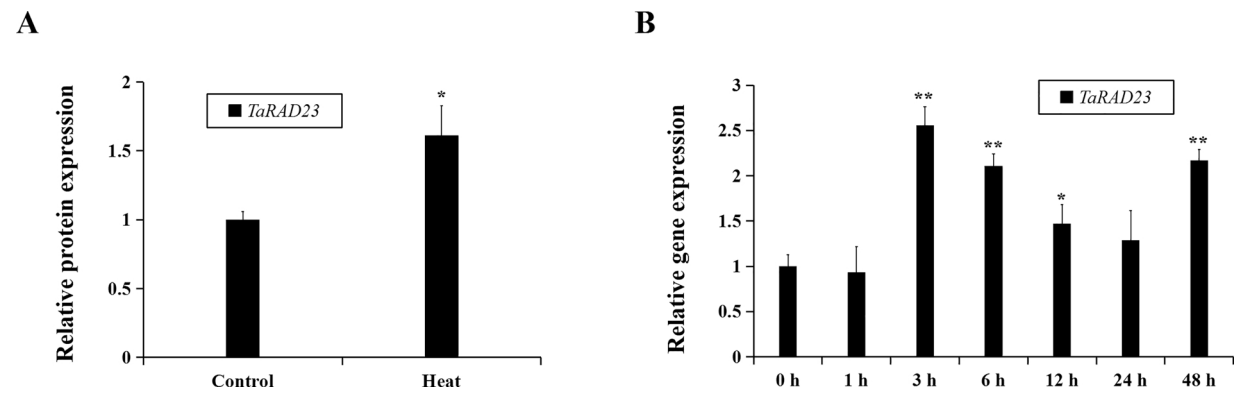

Fig. 1. The expression level of *TaRAD23* in response to heat stress (A) Relative protein abundance of TaRAD23 in the filling grains from plants grown under heat stress. (B) Relative gene expression of *TaRAD23* at different time points under heat stress. Total RNA prepared from filling grains at 15 days after anthesis was used to generate cDNA for qRT-PCR analysis. The relative expression level of each gene was normalized to *TaACTIN*. The ratio of gene/*TaACTIN* was averaged by values of three independent biological replicates, and the ratio of 0 h was set to a value of unity. Single and double asterisks above bars indicate significant differences between control and heat treatment at $P < 0.05$ level and $P < 0.01$ level (*t*-test) respectively.

3.2. The TaRAD23 sequence is highly conserved with AtRAD23B and AtRAD23 A

To examine whether the TaRAD23 is a conserved protein, we use the amino acid of TaRAD23 as the query to identify homologous proteins. More than 250 proteins were identified and annotated as DNA repair protein RAD23 or ubiquitin receptor RAD23, with the exception of a few putative RAD23 sequences and uncharacterized proteins (http://www.uniprot.org/). An amino acid sequence alignment of TaRAD23 and other annotated RAD23A/B/C/D proteins showed that they are highly conserved proteins and TaRAD23 was closely related to *A. thaliana* RAD23B and RAD23A, with amino acid sequence similarities of 66% and 64%, respectively (Fig. 2A). They all contain the signature UBL domain at their N-terminal region and two UBA domains in their central and C-terminal regions (Fig. 2B). Amino acid sequence alignments of the UBL domains also indicated that there are existing several conserved Lys residues and a hydrophobic patch (Leu-8, Ile-44, and Val-70). Besides, TaRAD23 has two UBA domains that containing the signature Met-Gly-Phe residues (MGF loop). Finally, TaRAD23 also contains a stress inducible 1 domain (STI, now named the heat-shock chaperone-binding domain).

3.3. Identification of the rad23a, rad23b T-DNA insertion mutants and 35S-TaRAD23/rad23b complementary lines

To investigate whether TaRAD23 and *A. thaliana* AtRAD23B and AtRAD23A are functionally conserved *in vivo*, *rad23b-1* (SALK_103707), *rad23b-2* (SALK_075940) and *rad23a* (SALK_066603) T-DNA insertion

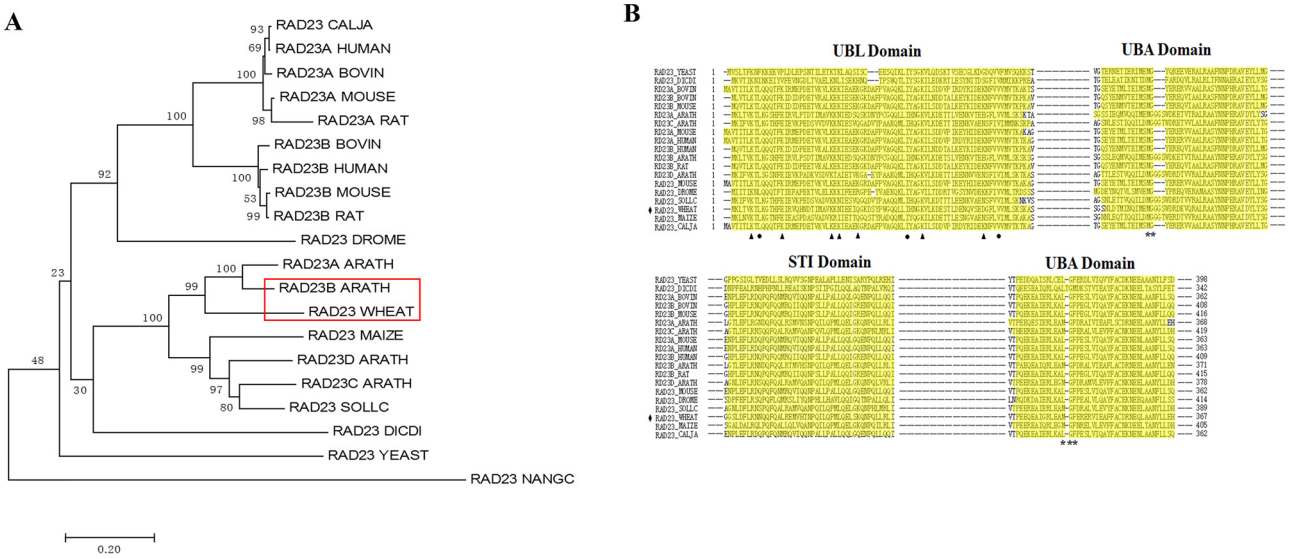

Fig. 2. Phylogenetic tree analysis and amino acid sequence alignment of TaRAD23 and other RAD23 proteins. (A) Phylogenetic tree analysis of TaRAD23 and RAD23 proteins from other species. The sequences used for phylogenetic analysis include the identified RAD23A/B/C/D from *Callithrix jacchus* (white-tufted-ear marmoset), *Homo sapiens* (human), *Bos taurus* (bovine), *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Drosophila melanogaster* (fruit fly), *Arabidopsis thaliana* (mouse-ear cress), *Zea mays* (maize), *Solanum lycopersicum* (tomato), *Dictyostelium discoideum* (slime mold), *Saccharomyces cerevisiae* (strain ATCC 204508/S288c) (baker's yeast), and *Nannochloropsis gaditana* (strain CCMP526) (green microalga). TaRAD23 from wheat and AtRAD23B from *A. thaliana* are marked with a red box. The phylogenetic tree was constructed with the Neighbor-Joining method by ClustalX 2.0 software. (B) Amino acid sequence alignment of TaRAD23 with RAD23 proteins from other species. Yellow shaded amino acids denote conserved domains: UBL domain (ubiquitin like domain), UBA domain (ubiquitin associated domain), and the STI domain (stress inducible 1 domain). The conserved lysine (Lys) residues in ubiquitin, used in poly-ubiquitin chain assembly are indicated by arrowheads. The residues forming the hydrophobic patch in ubiquitin (Leu-8, Ile-44, and Val-70), required for RPN10 binding, are highlighted by dots. Asterisks indicate residues of the hydrophobic MGF loop (Met-Gly-Phe), promoting the association of UBA and ubiquitin. TaRAD23 is marked with a diamond (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

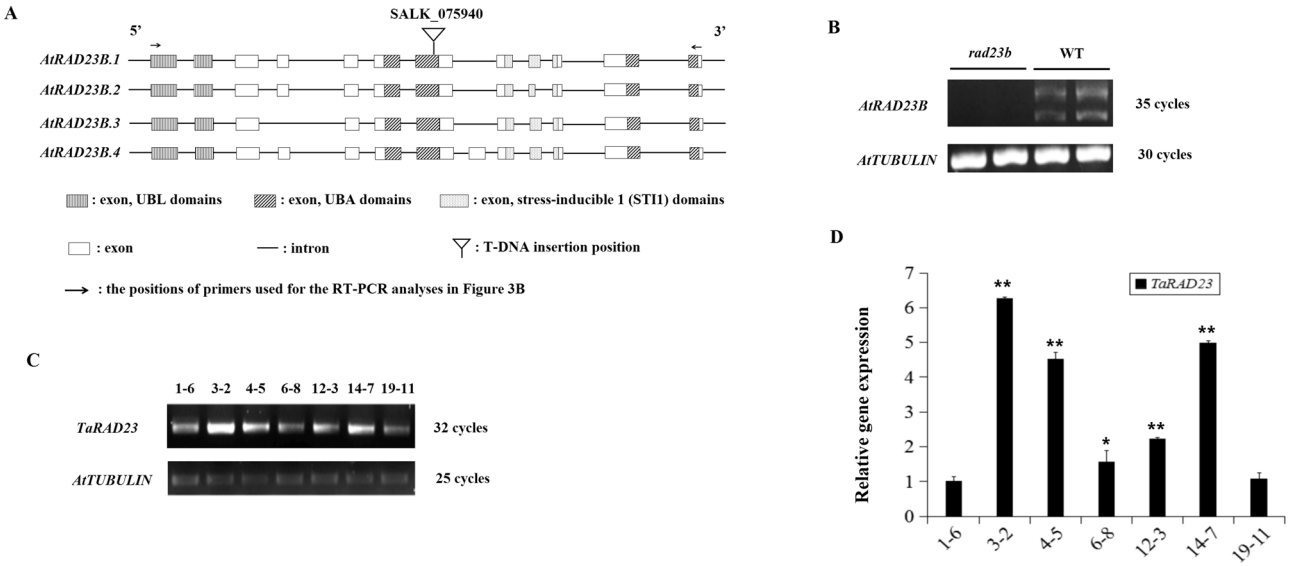

Fig. 3. Schematic overview of the *rad23b* T-DNA insertion mutant and expression level of *RAD23* in the mutant and complemented lines. (A) The T-DNA insertion site in *AtRAD23B*. The primers used for reverse transcription PCR (RT-PCR) were indicated by P1 and P2. (B) Expression level of *AtRAD23B* in wild type (WT) and *rad23b*. (C) Expression level of *TaRAD23* in *35S-TaRAD23/rad23b* complemented homozygous lines. (D) qRT-PCR analysis of *TaRAD23* expression in *35S-TaRAD23/rad23b* complemented homozygous lines. The *AtTUBULIN* gene was used as a control. Single and double asterisks above bars indicate significant differences between complementary line 1–6 and other complementary lines (3-2, 4-5, 6-8, 12-3, 14-7, 19-11) at $P < 0.05$ level and $P < 0.01$ level (*t*-test) respectively.

mutant seeds were ordered from the Nottingham Arabidopsis Stock Centre (NASC, http://nasc.life.nott.ac.uk/) and homozygous T-DNA insertions were identified by PCR (data not shown) and reverse transcription PCR (RT-PCR). When the homozygous mutants of *rad23b-1*, *rad23b-2* and *rad23a* T-DNA insertion mutant were grown under normal conditions, only *rad23b-2* showed multiple developmental defects compared with the WT (Fig. S1), thus we selected *rad23b-2* (abbreviated as *rad23b* in the following article) for further studies (Fig. 3A and B). We overexpressed *TaRAD23* in the *rad23b* mutant under the control of the constitutive 35S promoter, generating homozygous *35S-TaRAD23/rad23b* complementary lines. The expression level of *TaRAD23* in independent transgenic lines was examined by RT-PCR and qRT-PCR (Fig. 3C and D). Based on high expression of the *TaRAD23*, we selected 35S-*TaRAD23/rad23b* line 3-2 and 14-7 for subsequent analyses.

3.4. TaRAD23 of wheat rescues the developmental defects of rad23b in Arabidopsis

Consisting with Farmer et al. observation, *rad23a* null mutant didn't show obvious phenotype, but *rad23b* showed pleiotropic phenotypes, including significantly shorter primary root, fewer lateral roots (Fig. 4A– C), shorter plant height, shorter siliques and reduced seed numbers (Fig. 4D, E, G and H), as previous reports [48]. Furthermore, we found several novel phenotypes of *rad23b*, which grew more slowly with smaller cotyledon area (Fig.4I and K), but bolted earlier with fewer rosette leaves (Fig. 4J and L). Mature *rad23b* plants displayed longer carpopodiums (Fig. 4F and G), sometimes had more branches (Fig. 4D). Notably, constitutive expression of the *TaRAD23* of wheat driven by *35S* promoter restored *rad23b* to normal development as the WT (Fig. 4), which strongly suggested that TaRAD23 and AtRAD23B are highly conserved in function and evolution, and regulate a broad range of developmental processes in plant.

3.5. TaRAD23 can rescue the thermotolerant defects of rad23b

To investigate the role of *TaRAD23* and *AtRAD23B* in acquired thermotolerance, wild type (WT), *rad23b* and *35S-TaRAD23/rad23b* seedlings were exposed to HS and their ratio of green seedlings were calculated (Fig. 5A–C), the number of lateral roots were counted (Fig. 5D–F), as well as the length of hypocotyl were measured (Fig. 5G–I). Compared with WT, the *rad23b* knockout seedlings were more sensitive to HS and their ratio of green seedlings, the number of lateral roots, and the length of hypocotyl were almost as that of the *hot1* (a heat sensitive mutant) [56], however, transgenic plants rescued these thermotolerant defects or even displayed stronger thermotolerance than WT. Consequently, these results indicate that knockout of *AtRAD23B* in *rad23b* resulted in HS sensitivity and that the expression of the highly homologous wheat gene, *TaRAD23*, rescued the heat sensitive phenotype.

3.6. RAD23 mediate plant HS response process

To determine whether the *TaRAD23* gene involved in thermotolerance pathways by regulating expression of HSFs or HSPs, we performed qRT-PCR analysis of the expression levels of known HS responsive genes in the *rad23b* mutant and the *35S-TaRAD23/rad23b3-2* lines. The tested heat responsive genes were *HSFA2*, *HSFA3*, *HSHB1*, *HSP70*, *HSP90.1*, *ROF1*, *ROF2*, *HSP17.6*, *HSP18.2* and *HSA32* [2,57,58]. We detected lower expression of *HSFA2*, *HSP70*, *HSP90.1*, *ROF1*, *HSP17.6*, *HSP18.2* and *HSA32* in *rad23b* but and higher expression in *35S-TaRAD23/rad23b* (Fig. 6).

4. Discussion

We cloned a heat stress responsive gene *TaRAD23* in wheat, and found its expression was up-regulated by high temperature both at the RNA and protein level, suggesting its important role in wheat HSR. An amino acid sequence alignment of TaRAD23 and other annotated RAD23A/B/C/D proteins showed that they are highly conserved proteins that containing the signature UBL domain and UBA domains, and TaRAD23 was most homologous to *A. thaliana* RAD23B and RAD23A. Besides, the UBL domain of TaRAD23 also retained the several conserved Lys residues that involve in the formation of poly-Ub chain [59], and the hydrophobic patch (Leu-8, Ile-44, and Val-70) required for the binding of Ub to different Ub receptors [60], suggesting its conserved function. Furthermore, TaRAD23 has two UBA domains that contain a hydrophobic patch with the signature MGF loop. This patch has been

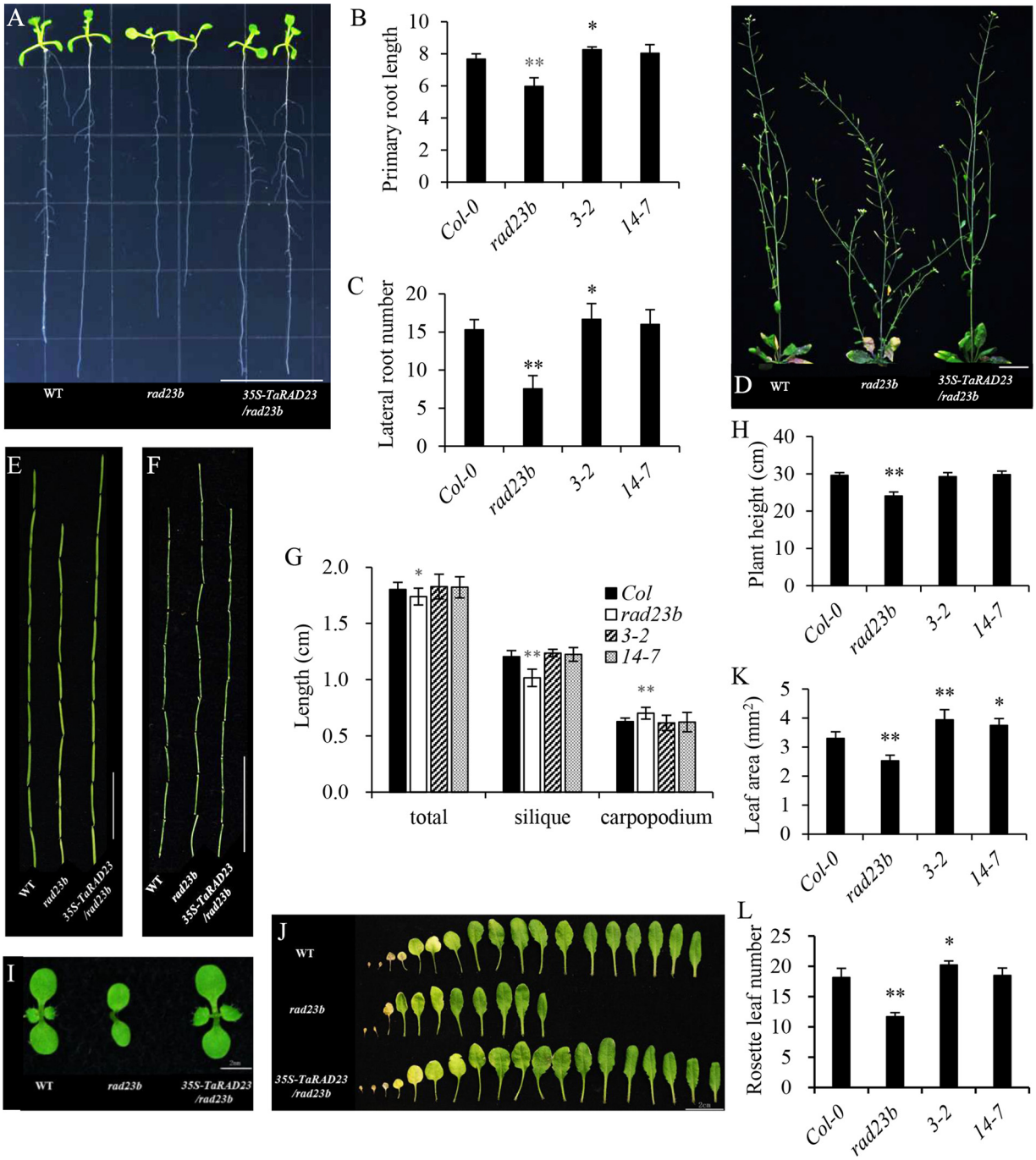

Fig. 4. Phenotypic analysis of wild type (WT), *rad23b* and *35S-TaRAD23/rad23b* plants. (A) Primary root and lateral root of 10 DAG (Days After Germination) seedlings. *35S-TaRAD23/rad23b* refer that the *rad23b* was complemented by *TaRAD23* with 35S promoter. WT, wild type *Col-0*. Bar = 2 cm. (B) and (C) Graphical representation of the length of primary root and number of lateral root in (A). *3-2* and *14-7* are two representative complementary lines of *rad23b*. (D) Plant height and branch of 11-week-old plants. Bar = 2 cm. (E) and (F) Siliques length and carpopodiums length of 10-week-old plants. Bar = 2 cm. (G) Graphical representation of length in (E) and (F). (H) Statistic data of plant height in (D). (I) Cotyledon phenotype of 10-day-old seedlings. Bar = 2 mm. (J) Rosette leaves of 36-day-old plants. Bar = 2 cm. All statistic data was acquired from nine plants for each genotype at least, Error bars indicate SD. Single and double asterisks above bars indicate significant differences between wild type and mutants or complemented lines at $P < 0.05$ level and $P < 0.01$ level (*t*-test) respectively.

demonstrated to interact with Ub in yeast RAD23 [61], suggesting that TaRAD23 also binds Ub. Finally, TaRAD23 also contains a STI domain, which consists of ~44 amino acids and has been implicated in the interaction between yeast RAD23 and the RAD4 sub-unit of the NEF2 DNA repair complex [62,63]. Taken together, amino acid sequence alignment suggests that the heat responsive TaRAD23 is a highly conserved protein and acts as a bridge between UPS and the heat stress response in wheat.

Multiple developmental defects were observed in the *rad23b* knockout mutant, including developmental retardation, earlier bolting with fewer rosette leaves, shorter primary root with fewer lateral roots, smaller siliques with longer carpopodiums and reduced seed set. Some of the phenotypes were similar to those described for animal homologs, such as mHR23B (a homolog of *S. cerevisiae* nucleotide excision repair factor Rad23B) [64]. However, the multiple developmental defects of the *rad23b* knockout mutant could be abrogated by expression of the

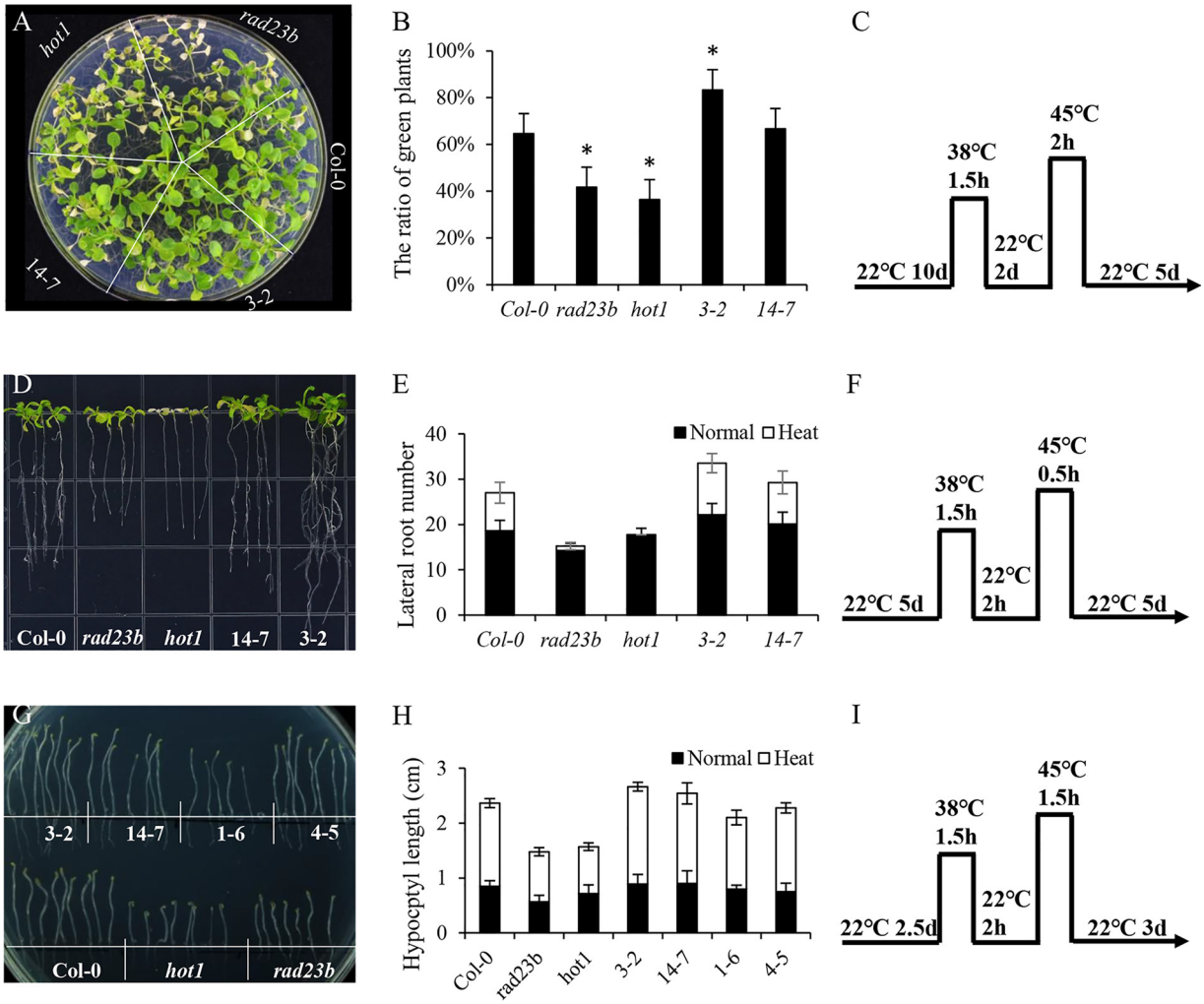

Fig. 5. RAD23 is required for long-term acquired thermotolerance. (A) Phenotype of the wild type (*Col-0*), *rad23b*, *hot1*, and *35S-TaRAD23/rad23b* plants (3-2, 14-7) after heat stress. (B) Statistic data of the ratio of green plants in (A). (C) The treatment conditions in (A). (D) Lateral root number after heat stress for the wild type (*Col-0*), *rad23b*, *hot1*, and *35S-TaRAD23/rad23b* plants (3-2, 14-7). (E) Statistic data of lateral root number in (D), lateral root number was measured before (black bars) and 5 d after (white bars) heat treatment. (F) The treatment conditions in (D). (G) Hypocotyl elongation after heat stress for the wild type (*Col-0*), *rad23b*, *hot1*, and *35S-TaRAD23/rad23b* plants (1–6, 3-2, 4-5, 14-7). (H) Statistic data of hypocotyl length in (G), hypocotyl elongation was measured before (black bars) and 3 d after (white bars) heat treatment. (I) The treatment conditions in (G). The experiments were repeated at least three times. Asterisks above bars indicate significant differences between wild type and mutants or complemented lines at $P < 0.05$ level (t-test) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

homologous wheat protein, TaRAD23. These findings suggest that RAD23 is evolutionarily and functionally highly conserved among eukaryotes, including plants and animals, and that it potentially plays crucial roles in cellular processes such as transcriptional regulation, DNA repair, cell cycle control, apoptosis and signal transduction, as well as protein quality control that regulates the destruction of misfolded and aberrant proteins under stress conditions.

In animals, the two mammalian homologs of yeast Rad23, HR23 A and HR23B are partially functionally redundant in the process of DNA repair, but they are multifunctional proteins, playing different roles in

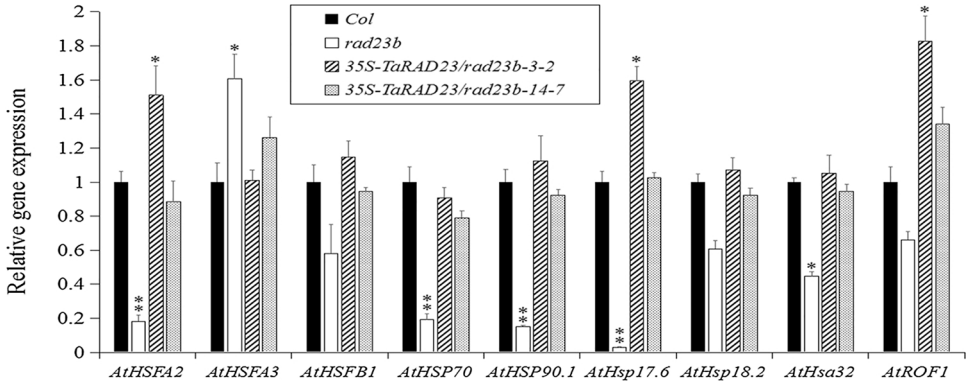

Fig. 6. Relative expression levels of heat responsive genes in wild type (*Col-0*), *rad23b* and *35S-TaRAD23/rad23b* plants. The relative expression level of each gene was normalized to *TaACTIN*. The ratio of gene/*TaACTIN* was averaged by values of three independent biological replicates, and the ratio of wild type was set to a value of unity. Asterisks above bars indicate significant differences between wild type and mutants or complemented lines, at $P < 0.05$ level (*t*-test).

other cellular processes [24]. In plants, it may be somewhat different since four RAD23 isoforms from *A. thaliana*, are clustered phylogenetically into two pairs, AtRAD23A and B (the amino acid identity/similarity is 81.2%/86.1%) and AtRAD23C and D (the amino acid identity/similarity is 62.3%/70.4%) [48]. When grown under a variety of conditions, single knockout mutants *rad23a*, *rad23c*, and *rad23d*, except for *rad23b*, have the normal fecundity and indistinguishable phenotypes compared with WT, indicating these proteins may have redundant functions. Only the *rad23b* knockout mutant showed abnormal developmental phenotypes similar to those seen in this study. Besides, they checked the response of *rad23b* seedlings to a series of treatments and found *rad23b* was only significantly more resistant to mitomycin C (MMC) compared with the WT, suggesting that AtRAD23B regulates cell division [48].

TaRAD23 was up-regulated by HS both at the RNA and protein levels, indicating its involvement in heat responses. When exposing to high temperature, we found *rad23b* seedlings were more sensitive to heat than the WT. However, they could be rescued by overexpression of TaRAD23, and the 35S-TaRAD23/*rad23b* lines tolerated severe HS as well as or even better than the WT plants. We concluded that loss of AtRAD23B caused HS sensitivity and that expression of the homologous protein TaRAD23 rescued the thermotolerant defects of the *rad23b* under HS. HSFs are key transcription factors in the HSR that can directly activate the expression of HSPs, sHSPs as well as other heat responsive proteins under heat stress, and over-expression of these genes can promote the ability of transgenic plants to enhance heat tolerance [65–71]. Then the expression levels of HSR related genes were investigated in *rad23b* and *35S-TaRAD23/rad23b* complementary lines to determine whether their expression levels were changed. The results indicated that the lower expression of the genes *HSFA2*, *HSP70*, *HSP90.1*, *ROF1*, *HSP17.6*, *HSP18.2* and *HSA32* in *rad23b* correlated with increased heat sensitivity, while higher expression of these genes in *35S-TaRAD23/rad23b* correlated with rescued or even stronger thermotolerance. These results suggest that the heat responsive *TaRAD23* may be involved in the regulatory network of plant HSR by regulating the expression level of *HSFA2* and its target genes.

RAD23 proteins are highly conserved among eukaryotes and play important roles in numerous cellular processes. These include working as shuttle proteins that target various ubiquitinated substrates to the proteasome for degradation, or to stabilize interacting proteins and prevent their proteasomal degradation. Comprehensive protein-protein interaction analyses are needed to determine candidate TaRAD23-interacting proteins related to the HS response, and further studies of these interactions will help elucidate the distinct role of the TaRAD23 regulatory mechanism in response to heat stress. We anticipate that this will provide useful information for generating thermotolerant wheat by bio-engineering or molecular breeding.

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Disclosures

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2018.04.028.

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